Kidney International, Vol. 38 (1990), pp. 1056–1060

Glomeruli synthesize nitrite in experimental nephrotoxic nephritis

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Glomeruli synthesize nitrite in experimental nephrotoxic nephritis. Activated macrophages synthesize nitric oxide (NO) from L-arginine. In culture, the major stable end product is nitrite (NO_2^-). Activated macrophages accumulate in glomeruli and are responsible for injury in experimental immune complex glomerulonephritis. We examined NO_2^- production by isolated glomeruli and urinary NO_2^- in accelerated nephrotoxic nephritis in the rat. Normal glomeruli did not produce NO_2^- spontaneously or when stimulated with lipopolysaccharide (LPS) (1 μg/ml) or A23187 (2 μg/ml). Cultured mesangial cells at first or seventh passage did not produce NO_2^- spontaneously or when stimulated. Nephritic glomeruli spontaneously produced NO_2^- at all times studied; this production was maximal at 24 hours after induction of glomerulonephritis (158.4 ± 8.4 nmol/48 hr/ml, $N = 3$). The production of NO_2^- was inhibited 75 to 100% by N^G-monomethyl-L-arginine (L-NMMA), and this inhibition was reversed by L-arginine, indicating NO_2^- production from L-arginine via NO. The production of NO_2^- was increased by LPS (1 μg/ml) at 2, 7 and 21 days. NO_2^- was undetectable in normal rat urine; however, it was present in urine of rats with glomerulonephritis (Day 0 to 1: 8161 ± 2605 nmol/24 hr, $N = 12$). The production of NO in nephritic glomeruli may have implications for both the mechanism of glomerular injury and glomerular hemodynamics.

Activated macrophages synthesize and release reactive nitrogen intermediates in culture; the predominant stable product is NO_2^- [1–3]. Although a biological role for NO_2^- synthesis has not yet been identified, its induction in vitro by LPS [1, 2], γIFN [2, 3], T cell-derived lymphokines and BCG infection [2] suggest a role in immunologically-mediated inflammation. Recent studies show that production of NO_2^- by activated macrophages occurs through synthesis of precursor nitric oxide (NO) derived from L-arginine [4–6]. NO is synthesized by vascular endothelium by a similar pathway [7].

Macrophages are involved in the pathogenesis of injury in immune complex glomerulonephritis [8]. We have recently reported that macrophages isolated from glomeruli in experimental glomerulonephritis have the characteristics of immune activation [9], namely enhanced superoxide generation, modulated eicosanoid synthesis and enhanced Class II MHC antigen expression. We have therefore sought evidence of spontaneous NO_2^- production in glomeruli in acute macrophage-associated glomerular inflammation. We chose for this study accelerated nephrotoxic nephritis, a model of glomerulonephritis where injury has previously been shown to be macrophage-dependent [8]. As normal glomeruli in the rat contain a small resident population of macrophages with a high percentage expressing Class II antigens [10], we also examined whether normal glomeruli produce NO_2^- either spontaneously or on stimulation.

Received for publication March 23, 1990
and in revised form June 20, 1990
Accepted for publication June 22, 1990

Methods

Materials

Glomerular and cell culture was carried out in Eagle's MEM without phenol red (Flow Laboratories, Irvine, UK) supplemented with sodium pyruvate (110 mg/liter), glucose (3.5 g/liter), glutamine (584 mg/liter), penicillin (50 U/ml), streptomycin (50 μg/ml), HEPES 15 mM and 10% fetal calf serum (FCS) (Flow Laboratories) pH 7.4. FCS contained 0.12 ng/ml endotoxin as determined by the supplier. Medium was prepared in deionized water (Milli-Q, Millipore). All supplements unless specified were from Sigma Chemical Co. (Poole, UK). All glassware was baked at 170°C for 4 hours to destroy endotoxin. Polymyxin B (Sigma Chemical Co.) 10 mg/ml was added to all washing buffers for glomerular isolation. LPS (*E. coli* 055:B5, Sigma), superoxide dismutase (SOD; ICN Biomedical, High Wycombe, UK), L-NMMA (Wellcome Research Laboratories, Beckenham, UK), L-arginine (Sigma) and A23187 (Sigma) were diluted in medium and used in incubations (see *NO_2^- production*). Griess reagent for NO_2^- assay was sulphamethazine 1% (Sigma), naphthyl ethylenediamine dihydrochloride 0.1% (Sigma) in 2.5% orthophosphoric acid.

Rats

Inbred Lewis rats from St. Mary's Hospital Medical School, weighing between 200 and 250 g were used.

Glomerular isolation

Glomeruli were isolated from normal or nephritic kidneys perfused in vivo with 50 ml sterile pyrogen-free saline (Phoenix Pharmaceuticals, Gloucester, UK) at room temperature, as previously described [11]. They were washed twice in culture medium before use.

Macrophage isolation from nephritic glomeruli

Isolated glomeruli were enzymically digested to single cell suspensions using trypsin, collagenase, DNase and EDTA

(Sigma) [12]. Cells were plated at 0.75 to 1.5 × 10^6/ml and washed after two hours to provide a population of glomerular macrophages as previously described [9]. Macrophages were enumerated as previously described [9].

Mesangial cell culture

Glomeruli were isolated from saline-perfused kidneys as above, and digested with Collagenase Type IV 750 U/ml (Sigma) for 20 minutes, washed in Hanks buffered salt solution, then plated at 600 glomeruli/ml in 16 mm fibronectin-coated wells. Glomeruli were cultured in RPMI 1640 (Sigma) plus 20% FCS, insulin 5 μg/ml, transferrin 5 μg/ml, sodium selenite 5 ng/ml, penicillin 50 U/ml and streptomycin 50 μg/ml (all supplements from Sigma). Mesangial cells were identified by positive staining with antimyosin antibody. For NO_2^- synthesis confluent primary and seventh passage cultures were used. Cultures were washed in NO_2^- medium then incubated for 48 hours in 0.5 ml medium per well. LPS (1 μg/ml), opsonized zymosan (1 mg/ml) (Sigma) [9], interleukin 1 (IL-1, 2.3 pg/ml, from Dr. A. Shaw, Glaxo, Geneva, Switzerland), and A23187 (1 μg/ml), were used as stimulants. All incubations were performed with and without L-NMMA 300 μM. Supernatants were harvested for NO_2^- assay. Wells were incubated with 200 μl 0.1 M NaOH and protein determined by the Bradford method [13].

NO_2^--production in vitro and assay

Isolated glomeruli were plated at 2000/ml in 16 mm plastic tissue culture wells (Nunc, Uxbridge, UK), and incubated for 48 hours at 37°C in 4% CO_2, with or without LPS 1 μg/ml, SOD 40 μg/ml, L-NMMA 300 μM or A23187 1 or 2 μg/ml. In one experiment on glomeruli isolated 24 hours after induction of GN, L-arginine 4.2 mM was used to demonstrate reversibility of L-NMMA inhibition; glomeruli were isolated and incubated with L-NMMA at concentrations of 25 nM or 50 nM in the presence or absence of L-arginine 4.2 mM. Supernatants were collected, centrifuged and stored at −20°C before assay.

NO_2^- concentration was determined by the Griess reaction [3]. One hundred microliters of culture supernatant or urine sample was mixed with an equal volume of Griess reagent in a 96-well plate and the absorbance was read at 550 nm (Titertek, Multiscan plate reader, Flow Laboratories). NO_2^- was determined using sodium nitrite as standard. Values were derived by subtracting background NO_2^- present in medium incubated for 48 hours without glomeruli or macrophages. For urine the value was corrected for the absorbance of urine diluted with water in place of Griess reagent.

Glomerulonephritis

Accelerated nephrotoxic nephritis [14] was induced by immunization of rats with 1 mg rabbit immunoglobulin (Sigma) in Freunds complete adjuvant (Sigma) i.p. one week prior to an i.v. subnephritogenic dose of rabbit anti-rat nephrotoxic globulin. Urine was collected from rats placed in metabolism cages. The detailed characteristics of this model using this nephrotoxic globulin preparation in our Lewis rats has recently been described [15]. Proteinuria (160 ± 19 mg/24 hr) is present at 24 hours. Renal histology shows a proliferative glomerulonephritis with linear deposition of rabbit and rat IgG, and rat C3 on glomerular capillary walls. The characteristics of leucocyte infiltration, when analyzed by cell isolation, are of neutrophil infiltration at four hours (mean 53 ± 17 neutrophils/glomerulus) and predominant macrophage infiltration from 24 hours onwards, (mean 94 ± 18 macrophages/gl at 24 hr). For the experiments described here glomeruli were isolated for NO_2^- assay at 4 and 24 hours, 2, 4, and 7 days, and three weeks after i.v. nephrotoxic globulin.

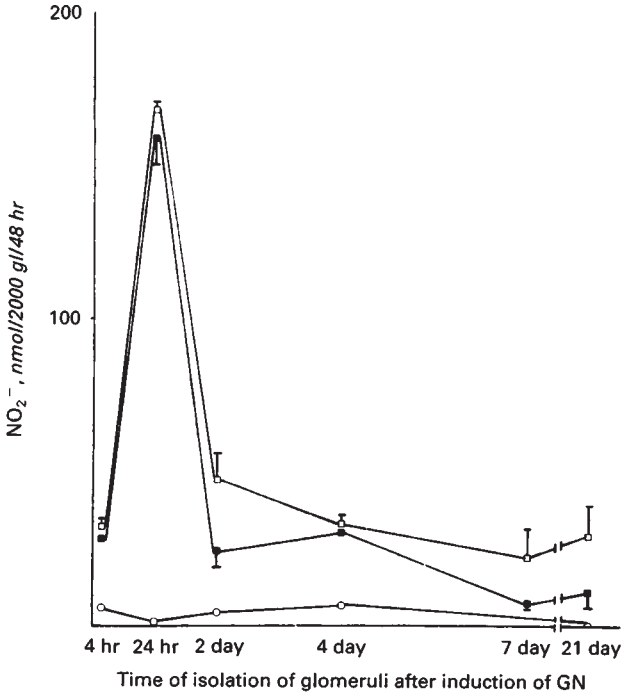

Fig. 1. *NO_2^- production by glomeruli (gl) isolated at various times after induction of GN.* Symbols are: (■) basal; (□) LPS 1 μg/ml; (○) L-NMMA 300 μM. Each point is mean ± SEM of 3 to 5 rats.

Results

Normal glomeruli

NO_2^- was not detected in the supernatants from normal glomeruli incubated under basal conditions or with A23187 (2 μg/ml) or LPS (1 μg/ml); lower limit of detection 1.5 nmol/ml/48 hr.

Mesangial cells

NO_2^- was not detected in supernatants from mesangial cell cultures under basal or stimulated conditions (mean cell protein concentration 47 μg/well).

Nephritic glomeruli

NO_2^- was present in supernatants of glomeruli cultured under basal conditions at all times examined (Fig. 1). Production was maximal 24 hours after induction of glomerulonephritis (158 ± 8 nmol/ml/48 hr). There was an increase in NO_2^- when LPS was added to the medium of glomeruli which reached significance in samples 2, 7 and 21 days after induction of glomerulonephritis ($P < 0.05$, Student's paired sample t-test; Fig. 1). Incubation with L-NMMA (300 μM) inhibited NO_2^- production by between 75 and 100% in both basal conditions (Fig. 1) and with LPS stimulation. About 60% of the NO_2^- production occurred in the first 24 hours of culture (Fig. 2).

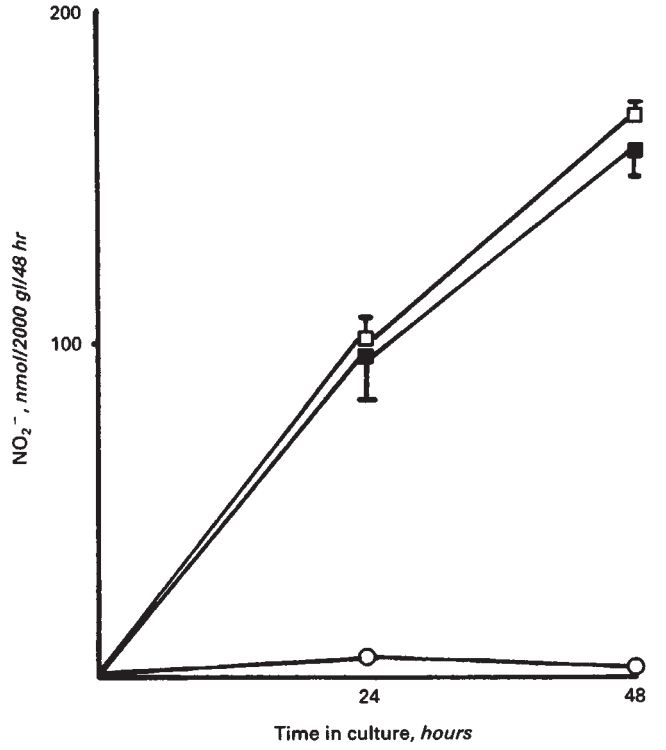

Fig. 2. *NO_2^- production over time by glomeruli (gl) isolated 24 hours after induction of GN.* Symbols are: (■) basal; (□) LPS 1 μg/ml; (○) L-NMMA 300 μM. Each point is mean ± SEM of 3 rats.

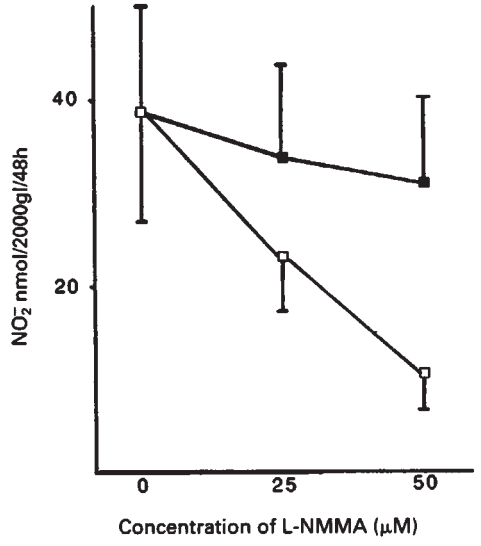

Fig. 3. *Effect of L-arginine on inhibition of NO_2^- production by L-NMMA.* Glomeruli (gl) were isolated 24 hours after induction of GN. Symbols are: (■) L-arginine 4.2 mM; (□) no added L-arginine. Each point is mean ± SEM of four rats.

L-arginine 4.2 mM significantly reduced the inhibitory effect of L-NMMA 50 μM (Fig. 3; $P < 0.05$, Student's paired sample *t*-test). Addition of SOD to cultures had no effect on NO_2^- production.

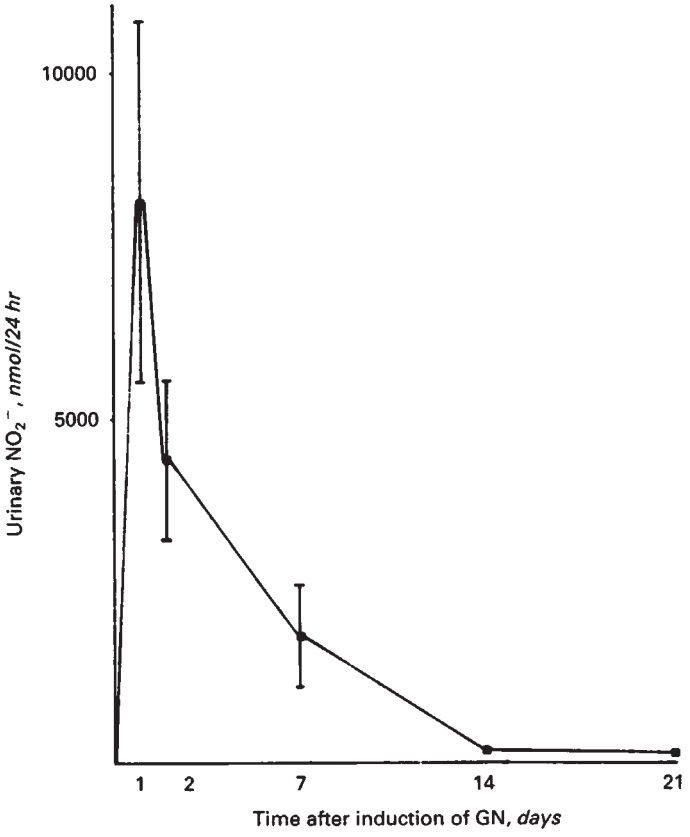

Fig. 4. *NO_2^- in rat urine collected over 24 hours at various times after induction of GN.* Each point is mean ± SEM (day 1, $N = 12$; day 2, $N = 13$; other points $N = 4$).

Isolated glomerular macrophages

Adherent cells from glomeruli isolated at 24 hours produced 30.7 ± 2.8 nmol NO_2^-/48 hr/10^6 macrophages plated ($N = 2$). This was inhibited by greater than 90% by L-NMMA.

Urinary NO_2^-

NO_2^- was undetectable in normal rat urine (Fig. 4). In urine from nephritic rats it was maximal from day 0 to 1 (8161 ± 2605 nmol/24 hr, $N = 12$) and was still raised after three weeks; urine volumes (mls/24 hr; mean ± SEM) were 22 ± 4 (day 1), 10 ± 2 (day 2), 8 ± 1 (day 7), 9 ± 1 (day 14) and 18 ± 3 (day 21).

Discussion

We demonstrate here that inflamed glomeruli in nephrotoxic serum nephritis produce NO_2^- in culture. This production occurs without further stimulation. NO_2^- is also detectable in large amounts in the urine of nephritic rats. In contrast NO_2^- is not spontaneously synthesized by normal glomeruli and no NO_2^- is detectable in the urine of normal rats.

This is the first demonstration of NO_2^- production at a site of immunologically induced inflammation. Synthesis of oxides of nitrogen represents an unusual pathway of oxidation in mammals; this pathway has been described in three mammalian cell types which are present in glomeruli in our model of immune-induced inflammation: macrophages [1–3], neutrophils [16], and endothelial cells [7]. In the earliest phase of injury induced by

nephrotoxic globulin (within 2 hours) there is ultrastructural evidence of endothelial injury [17]. Neutrophil infiltration occurs within the first 24 hours and from 24 hours there is a major infiltration by macrophages [15], which persists at least up to the time of our final analysis at three weeks.

We found that NO_2^- generation was markedly inhibited by the methylated arginine derivative L-NMMA and this inhibition could be reversed by L-arginine, strong evidence that the NO_2^- is derived from L-arginine as has been shown for macrophages [18–20] and endothelial cells [21]. NO_2^- is derived from one of the two-terminal guanido nitrogens of arginine via the nitric oxide radical with the concomitant generation of L-citrulline [4, 20]. NO_3^- is also an end product of NO metabolism [4] but only NO_2^- is measured by our assay. Marletta et al [4] have put forward reasons why the ratio of NO_2^- to NO_3^- may be expected to vary with the concentration of NO. Incubation with SOD did not affect NO_2^- levels; a result similar to that found with isolated macrophage cell lines [20].

Macrophages are the most likely source of most of the NO_2^- we have detected. Neutrophils are only present in small numbers by 24 hours [15] when NO_2^- production is at its peak; this peak coincides with the main influx of macrophages. The macrophages in glomeruli in nephrotoxic nephritis are of activated type. We have shown previously [9] that macrophages isolated from nephritic glomeruli have markers of immune activation such as high Ia antigen expression. NO_2^- production by macrophages is related to activation [2, 3]. Macrophages elicited with inflammatory stimuli such as thioglycollate broth or periodate do not spontaneously produce NO_2^- but do so after further stimulation with γIFN or with αIFN or βIFN in combination with LPS [3]. Under these circumstances macrophages will continue to generate NO_2^- over one to three days of culture without further stimulus. NO_2^- is also produced by macrophages generated by an immune stimulus such as BCG [2]. That macrophages are, at least in part, the source of NO_2^- production by nephritic glomeruli is supported by the NO_2^- production by macrophages isolated from nephritic glomeruli.

Endothelial cells also produce NO in response to stimuli such as bradykinin and A23187, and after A23187 the release is protracted [7]. We found no stimulation of production of NO_2^- by A23187 applied to normal glomeruli; however, we cannot exclude the possibility that some of the NO_2^- we have detected may arise from endothelial cells damaged by the inflammatory response. The lack of production by normal glomeruli also suggests that the small number of resident glomerular macrophages [10] are either too few or not susceptible to stimulation, and further that normally at least, mesangial cells do not produce NO_2^-: neither did mesangial cells in culture produce NO_2^-. However, we still cannot exclude possible production by mesangial cells stimulated by inflammation.

We found markedly raised levels of NO_2^- in the urine at the height of glomerular injury. As far as we are aware raised urinary NO_2^- has not been noted in renal disease. Others have studied urinary excretion of total NO_2^- plus NO_3^- by reducing NO_3^- to NO_2^- before measuring NO_2^-, and have shown that NO_2^-/NO_3^- in urine is raised after injection of LPS or mycobacteria in mice [1, 22] and during fever in a human [22]. NO_2^- is rapidly destroyed in the blood [23], as a result of its interaction with oxyhemoglobin to produce methemoglobin and NO_3^- [24]. It is therefore unlikely that the NO_2^- we have found in urine is a reflection of systemic production.

Although oxides of nitrogen are important mediators in the blood vessel wall [25–27] and a general cell-cell regulatory function has been suggested for the L-arginine, NO pathway [28], the role of this metabolic pathway in inflammation is unknown. It has been shown to be responsible for tumor cell cytotoxicity by macrophages [18, 29]; in tumor cells NO causes inhibition of DNA synthesis and mitochondrial respiration [29]. As macrophages are responsible for glomerular injury in this model of glomerulonephritis [8], generation of NO is one way in which they might damage glomerular cells. Furthermore, the vasodilator action of NO may have a role in preserving glomerular blood flow, as the acute reduction in single nephron glomerular filtration rate known to occur immediately on fixation of nephrotoxic globulin [30] reverses in the autologous phase of injury [31]. Our data demonstrate a novel mediator system in glomerular pathophysiology. Further exploration of the role of reactive nitrogen products in glomerulonephritis may offer new insights into possible pharmacological manipulation.

Acknowledgments

Part of this work was presented in abstract form at the British Pharmacological Society meeting, January 1990. Interleukin-1 used in this study was a gift from Dr. A. Shaw, Glaxo, Geneva, Switzerland. Miss Helen Wang is thanked for technical assistance.

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